

## Expression of fibroblast growth factors (Fgfs) in murine tooth development

Thantrira Porntaveetus,<sup>1</sup> Yoko Otsuka-Tanaka,<sup>1</sup> M. Albert Basson,<sup>1</sup> Anne M. Moon,<sup>2</sup> Paul T. Sharpe<sup>1</sup> and Atsushi Ohazama<sup>1</sup>

<sup>1</sup>Department of Craniofacial Development, Dental Institute, King's College London, Guy's Hospital, London, UK

<sup>2</sup>Department of Neurobiology and Anatomy, University of Utah, Salt Lake City, UT, USA

### Abstract

Fgf signalling is known to play critical roles in tooth development. Twenty-two Fgf ligands have been identified in mammals, but expression of only 10 in molars and three in the incisor loop stem cell region have been documented in murine tooth development. Our understanding of Fgf signalling in tooth development thus remains incomplete and we therefore carried out comparative *in situ* hybridisation analysis of unexamined Fgf ligands (eight in molars and 15 in cervical loops of incisors; *Fgf11–Fgf14* were excluded from this analysis because they are not secreted and do not activate Fgf receptors) during tooth development. To identify where Fgf signalling is activated, we also examined the expression of *Etv4* and *Etv5*, considered to be transcriptional targets of the Fgf signalling pathway. In molar tooth development, the expression of *Fgf15* and *Fgf20* was restricted to the primary enamel knots, whereas *Etv4* and *Etv5* were expressed in cells surrounding the primary enamel knots. *Fgf20* expression was observed in the secondary enamel knots, whereas *Fgf15* showed localised expression in the adjacent mesenchyme. *Fgf16*, *Etv4* and *Etv5* were strongly expressed in the ameloblasts of molars. In the incisor cervical loop stem cell region, *Fgf17*, *Fgf18*, *Etv4* and *Etv5* showed a restricted expression pattern. These molecules thus show dynamic temporo-spatial expression in murine tooth development. We also analysed teeth in *Fgf15*<sup>-/-</sup> and *Fgf15*<sup>-/-</sup>;*Fgf8*<sup>+/-</sup> mutant mice. Neither mutant showed significant abnormalities in tooth development, indicating likely functional redundancy.

**Key words:** cervical loop; epithelium; Fgf ligand; *Fgf15* mutant; *Fgf15/Fgf8* mutant; mesenchyme; primary enamel knot; secondary enamel knot; tooth development.

### Introduction

Fibroblast growth factor (Fgf) signalling has been shown to play essential roles in regulating many biological processes including mitogenic, chemotactic and angiogenic activity and wound healing (Powers et al. 2000; Nie et al. 2006; Mason, 2007). It is known that Fgf signalling controls the development of many craniofacial organs: brain, palate, salivary glands, olfactory, eye and ear (Min et al. 1998; Sekine et al. 1999; De Moerlooze et al. 2000; Mason, 2007).

Teeth are craniofacial organs that are known to be regulated by Fgf signalling during their development (Thesleff et al. 1995; Pispá & Thesleff, 2003; Tucker & Sharpe, 2004;

Mikkola, 2007). One of the roles of Fgf signalling in tooth development is for bud formation, since tooth development is arrested as thickening epithelium in Fgf receptor (*Fgfr*) *2IIIb* mutant mice (De Moerlooze et al. 2000; Hosokawa et al. 2009). Fgf signalling is also important for continuous growth of murine incisors, as *Fgf10* mutant mice lack cervical loop formation, an epithelial stem-cell niche for providing ameloblast precursors (Harada et al. 2002). Fgf signalling has also been shown to be involved in determining tooth type by inducing homeobox gene expression in presumptive tooth mesenchyme at early stages of development (Tucker et al. 1998). Fgf signalling thus regulates multiple aspects of tooth development (Supporting Information Table S1 summarises the tooth phenotypes caused by targeted disruption of Fgf signalling-related molecules).

Although 22 Fgf ligands have been identified in mammals, only 10 (*Fgf1–Fgf10*) and three (*Fgf3*, *Fgf9* and *Fgf10*) Fgf ligands have been documented in murine molar tooth development and murine incisor cervical loop region development, respectively (Kettunen & Thesleff, 1998; Harada et al. 1999; Kettunen et al. 2000; Unda et al. 2001; Wang

### Correspondence

Atsushi Ohazama, Department of Craniofacial Development, Dental Institute, King's College London, Floor 27, Guy's Hospital, London Bridge, London, SE1 9RT UK. T: + 44 20 71881795; F: + 44 20 71881674; E: atsushi.2.ohazama@kcl.ac.uk

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et al. 2007; Klein et al. 2008). Our understanding of Fgf signalling in tooth development thus remains incomplete.

A comprehensive expression analysis of all unexamined Fgfs in the tooth development was carried out to obtain a more complete understanding of the role of Fgf signalling in tooth development. The expression patterns of unexamined Fgf ligands (eight in the molar and 15 in the cervical loop region) and two Fgf signalling-related molecules (*Etv4* and *Etv5*) were mapped by *in situ* hybridisation in mouse embryonic tooth germs. *Fgf15*, *Fgf16*, *Fgf17*, *Fgf18*, *Fgf20*, *Fgf21*, *Etv4* and *Etv5* showed dynamic temporo-spatial expression, whereas the expression of *Fgf19* or *Fgf22* could not be detected in tooth development. Among the Fgf ligands examined, *Fgf15* showed restricted expression patterns in tooth development, and therefore tooth development in *Fgf15*<sup>-/-</sup> and *Fgf15*<sup>-/-</sup>;*Fgf8*<sup>+/-</sup> mutant mice was examined. Neither mutant showed significant abnormalities in tooth development, indicating likely functional redundancy.

## Materials and methods

### Production and analysis of transgenic mice

*Fgf15*<sup>-/-</sup> mice were produced as described by Wright et al. (2004) and Vincentz et al. (2005). *Fgf8*<sup>+/-</sup> mice were produced as described by Moon & Capecchi (2000). Mutant and littermate heads were fixed in 4% paraformaldehyde (PFA), wax-embedded and serially sectioned for histological analysis. Eight *Fgf15*<sup>-/-</sup> and eight *Fgf15*<sup>-/-</sup>;*Fgf8*<sup>+/-</sup> mice were examined in this study.

### *In situ* hybridisation

Radioactive *in situ* hybridisation with [<sup>35</sup>S]UTP-labeled riboprobes was carried out as described previously (Ohazama et al., 2008). Briefly, CD1 and mutant mouse heads were fixed in 4% PFA, wax-embedded and serially sectioned at 7 µm. Decalcification using 0.5 M EDTA (pH 7.6) was performed after fixation of newborn mice. Sections were split over 5–10 slides and prepared for radioactive *in situ* hybridisation. Radioactive *in situ* hybridisation with [<sup>35</sup>S]UTP-labeled riboprobes was carried out as described by Wilkinson (1995), with modifications. Briefly, sections were floated onto TESPA(3-aminopropyltriethoxysilane)-coated slides. The slides were pretreated with 5 mg mL<sup>-1</sup> proteinase K and 0.25% (vol/vol) acetic anhydride to reduce background. Hybridisation was carried out overnight in a humidified chamber at 55 °C. The slides were then washed twice at high stringency in 2 × standard saline citrate (SSC), 50% formamide, 10 mM dithiothreitol (DTT) at 65 °C for 20 min and treated with 40 µg mL<sup>-1</sup> RNase A for 30 min at 37 °C to remove any non-specifically bound probe. The high stringency washes (at 65 °C in 2 × SSC, 50% formamide, 10 mM DTT) were repeated, followed by a further wash at 65 °C in 0.1 × SSC, 10 mM DTT. The sections were then washed in 0.1 × SSC at room temperature and dehydrated through 300 mM ammonium acetate in 70% ethanol, 95% ethanol and absolute ethanol. The slides were air-dried and dipped in Ilford K.5 photographic emulsion. Autoradiography was performed by exposing the sections in a light-tight box at

4 °C for 10–14 days. Slides were developed using Kodak D19, fixed in Kodak UNIFIX, counterstained with malachite green or haematoxylin, and mounted with DePex (BDG). For photography, in some sections the darkfield images were inverted, artificially stained red, and combined with the brightfield image using ADOBE PHOTOSHOP. The plasmids used to prepare the radioactive antisense probes in this study have previously been described and were generated from mouse cDNA clones *Fgf1*, *Fgf2*, *Fgf5*, *Fgf6*, *Fgf7*, *Fgf15*, *Fgf16*, *Fgf17*, *Fgf19*, *Fgf20*, *Fgf21* (Yaguchi et al. 2009), *Fgf4* (Sun et al. 2000), *Fgf8* (Mahmood et al. 1995), *Fgf18* (Ohbayashi et al. 2002) and *Fgf22* (Umemori et al. 2004).

## Results and Discussion

### Expression of Fgf ligands and Fgf signalling molecules in tooth development

Teeth develop as a result of sequential and reciprocal interactions between oral epithelium and mesenchyme. The first morphological sign of tooth development is a narrow band of thickened epithelium on the developing jaw primordia. The thickened epithelium progressively takes the form of bud, cap and bell configurations as differentiation proceeds. Subsequently, epithelial cells and mesenchymal cells (dental papilla) differentiate into enamel-producing ameloblasts and dentine-producing odontoblasts, respectively.

Mice have only one incisor and three molars in each jaw quadrant, which are divided by a large edentulous region (diastema). It is known that mice lost 'diastema' teeth during evolution and despite the lack of these teeth in the diastema, two rudimentary epithelial buds (called MS and R2) form as vestigial tooth germs in the murine diastema. R2 subsequently integrates into the first molar tooth germs during early stages of development, whereas MS is removed by apoptosis (Ahn et al. 2010; Prochazka et al. 2010).

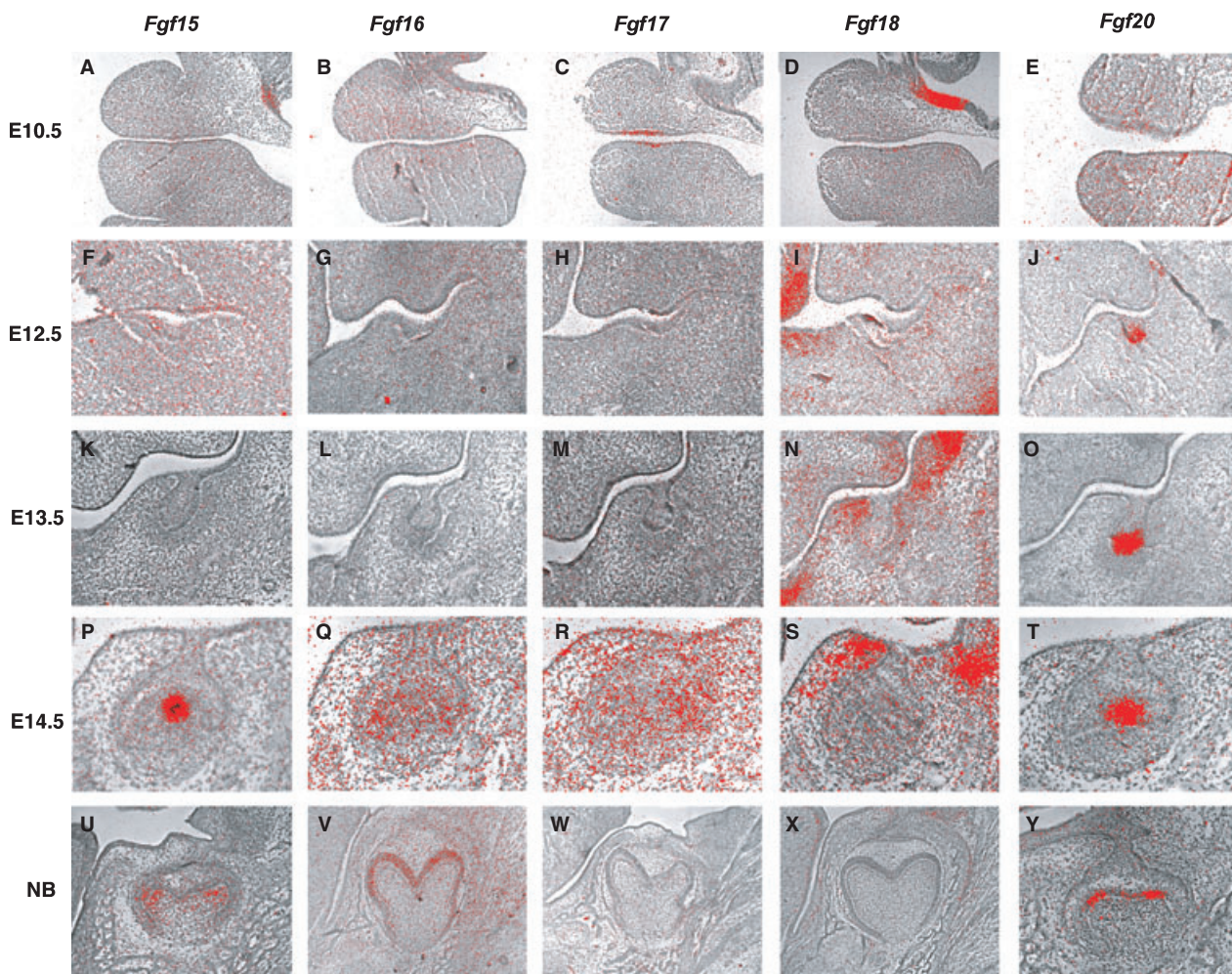
We carried out comparative *in situ* hybridisation analysis of molar tooth development at E10.5, E12.5, E13.5, E14.5 and newborn mice. This period encompasses molar tooth development from initiation to the onset of cytodifferentiation.

Fgf ligands can be subdivided broadly into seven subfamilies based on sequence homology. The members of the *Fgf11* subfamily (*Fgf11*–*Fgf14*) were excluded from this analysis because they are not secreted and do not activate Fgf receptors, but instead are localised to the cell nucleus and have intracellular function (Powers et al. 2000; Zhang et al. 2006; Mason, 2007).

#### *Initiation of tooth development (E10.5)*

Initiation begins before the tooth anlagen is morphologically visible. The first signals are derived from tooth presumptive epithelium at E9.5 (Ferguson et al. 2000). The first visible signs of tooth development cannot be recognised at E10.5.

Strong *Fgf17* expression was observed in presumptive tooth epithelium (Fig. 1C). *Fgf8* and *Fgf9* were also



**Fig. 1** Expression of Fgf ligands in molar tooth development. *In situ* hybridisation of *Fgfs* on frontal head sections at E10.5 (A–E), E12.5 (F–J), E13.5 (K–O), E14.5 (P–T) and E18.5 (U–Y).

expressed in presumptive tooth epithelium, whereas *Fgf10* expression was found in both epithelium and mesenchyme at this stage (Kettunen & Thesleff, 1998). *Fgf17* was expressed in the presumptive molar region but not in the presumptive incisor region at E10.5, suggesting that *Fgf17* expression is very similar to *Fgf8* at this stage (data not shown). Both *Fgf8* and *Fgf17* belong to the *Fgf8* subfamily. *Fgf17* may thus regulate the determination of tooth type, as *Fgf8* is known to play critical roles in determining tooth type (Neubuser et al. 1997; Tucker et al. 1998). *Fgf15*, *Fgf16*, *Fgf18*, *Fgf20*, *Fgf21*, *Fgf22* and *Fgf23* showed no expression in presumptive tooth regions at E10.5 (Fig. 1–A,B,D; data not shown).

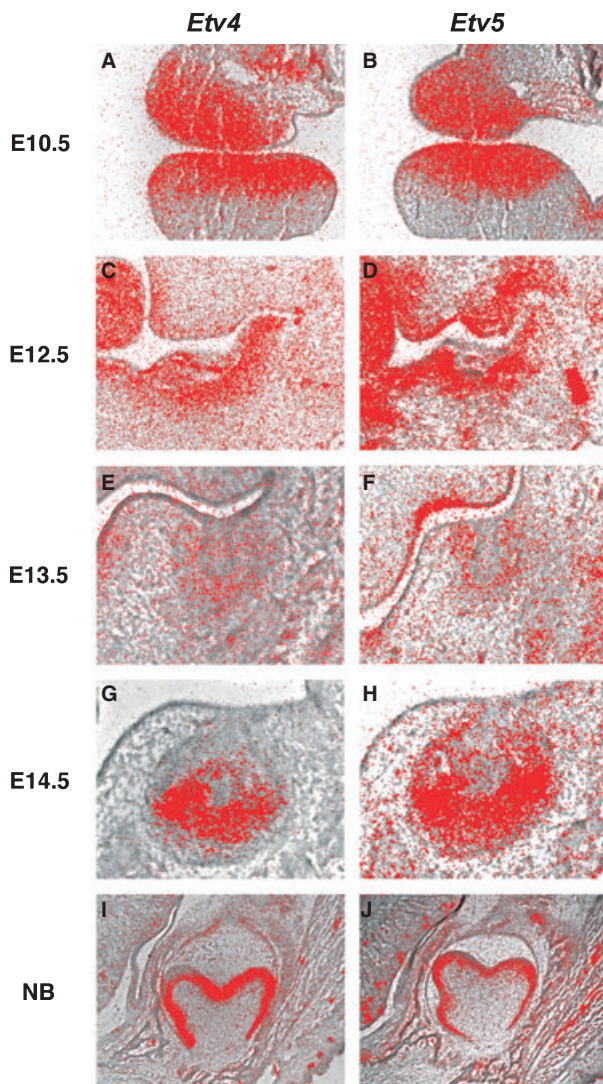
We next assayed the expression of the ETS-related factor genes *Etv4* (*Pea3*) and *Etv5* (*Erm*), which are considered to be transcriptional targets of the Fgf signalling pathway (O'Hagen & Hassell 1996; Roehl & Nusslein-Volhard, 2001). Expression of both genes was observed in epithelium and mesenchyme (Fig. 2A,B). Fgf signalling is transduced

through a family of four transmembrane receptor tyrosine kinases in all vertebrates (Powers et al. 2000; Mason, 2007). It has been shown that *Fgfr1IIIc* (receptor for Fgf8, Fgf17 and Fgf20) and *Fgfr2IIIb* (receptor for Fgf10) are expressed in tooth mesenchyme and epithelium, respectively, at this stage (Kettunen et al. 1998; Mason, 2007). It is thus likely that, at this stage, Fgf signalling is activated in epithelium by Fgf10 through *Fgfr2IIIb* and in mesenchyme by Fgf8, Fgf17 and Fgf20 through *Fgfr1IIIc*.

#### *Thickening tooth epithelium (E12.5)*

Thickening of the oral epithelium takes place from E12. The anterior limit of thickened epithelium is the vestigial tooth germ (MS), which is more prominent than the posterior part of thickened epithelium. *Shh* expression is only observed in MS epithelium at this stage (Ahn et al. 2010; Prochazka et al. 2010).

*Fgf15* was weakly expressed at the lingual side of the thickened tooth epithelium, whereas strong *Fgf20* expres-



**Fig. 2** *Etv4* and *Etv5* expression in molar tooth development. *In situ* hybridisation of *Etv4* and *Etv5* on frontal head sections at E10.5 (A,B), E12.5 (C,D), E13.5 (E,F), E14.5 (G,H) and E18.5 (I,J).

sion was observed at the tip of MS (Fig. 1F,J). *Fgf18* was weakly expressed at the buccal side of tooth mesenchyme (Fig. 1I). The expression of *Fgf16*, *Fgf17*, *Fgf21*, *Fgf22* and *Fgf23* could not be detected in tooth germs at this stage (Fig. 1G, 1H; data not shown). Both *Etv4* and *Etv5* were strongly expressed in mesenchyme, whereas only weak expression was observed in epithelium (Fig. 2C,D). It has been shown that *Fgf10* is faintly expressed in the mesenchyme, whereas *Fgf8* and *Fgf9* expression is observed in epithelium at this stage (Kettunen & Thesleff, 1998; Kettunen et al. 2000). It is also known that *Fgfr1IIIc* (receptor for Fgf8, Fgf15 and Fgf20) is expressed in mesenchyme and that *Fgfr2IIIb* (receptor for Fgf10 and Fgf15) expression is found in epithelium at this stage (Kettunen et al. 1998; Mason, 2007). It is likely that Fgf signalling is activated in epithelium by Fgf10 and Fgf15 through *Fgfr2IIIb*, and in mesenchyme

by Fgf8, Fgf15 and Fgf20 through *Fgfr1IIIc*. In *Fgfr2 IIIb* mutant mice, tooth development is arrested as thickened epithelium, suggesting that Fgf10 and Fgf15 play critical roles in the transition to the bud stage (De Moerloose et al. 2000; Hosokawa et al. 2009).

#### *Bud stage (E13.5)*

By E13.5 the tooth epithelium invaginates into underlying mesenchyme to form the epithelial bud. R2 buds become prominent posterior to MS expressing *Shh*, whereas MS loses *Shh* expression at this stage (Ahn et al. 2010; Prochazka et al. 2010).

*Fgf20* expression was found at the posterior part of tooth germs, whereas the anterior part of tooth germs (MS or R2) showed no expression (Fig. 1O; Supporting Information Fig. S1). Expression of *Fgf18* (Fig. 1N) was observed at the buccal and lingual sides of tooth mesenchyme but was absent from mesenchyme underneath bud epithelium (Fig. 1N). *Fgf15*, *Fgf16*, *Fgf17*, *Fgf21*, *Fgf22* and *Fgf23* showed no expression in tooth germs at E13.5 (Fig. 1K,L,M; data not shown). Weak expression of *Etv4* and *Etv5* was observed in both epithelium and mesenchyme (Fig. 2E,F; Klein et al. 2006). *Fgf8* expression was restricted to the oral part of epithelium, whereas *Fgf3* was weakly expressed in both epithelium and mesenchyme at this stage (Kettunen & Thesleff, 1998; Kettunen et al. 2000). It has also been shown that strong *Fgf9* expression and weak *Fgf4* and *Fgf3* expression are observed at the tip of bud epithelium, similar to *Fgf20* expression (Fig. 1O; Kettunen & Thesleff, 1998). *Fgf10* is expressed in the mesenchyme on the buccal and lingual sides of tooth germs at this stage (Kettunen et al. 2000). *Fgfr1IIIc* (receptor for Fgf4, Fgf8 and Fgf20) and *Fgfr2IIIc* (receptor for Fgf4, Fgf8, Fgf9, Fgf16, Fgf18 and Fgf20) expression are found in mesenchyme, whereas *Fgfr2IIIb* (receptor for Fgf3 and Fgf10) is expressed in epithelium at this stage (Kettunen et al. 1998; Mason, 2007). It is likely that Fgf signalling is activated in epithelium by Fgf3 and Fgf10 through *Fgfr2IIIb*, and in mesenchyme by Fgf4, Fgf8, Fgf9, Fgf18 and Fgf20 through *Fgfr1IIIc* and *Fgfr2IIIc*.

#### *Cap stage (E14.5)*

By E14.5 the bud basal epithelium develops into the internal and the external (outer) enamel epithelium, and the mesenchyme develops into the dental papilla and the dental follicle. The temporal and spatial folding of the internal enamel epithelium is required for formation of cusps. Primary enamel knots are transient populations of cells in the center of the invaginating dental epithelium originally identified in histological sections of cap-stage tooth germs (MacKenzie et al. 1992; Jernvall et al. 1998). The primary enamel knots are located at the posterior tooth germs that show *Shh* expression; the anterior part of tooth germs (MS and R2) exhibit no *Shh* expression at this stage (Ahn et al. 2010; Prochazka et al. 2010).

*Fgf15* and *Fgf20* expression was found at the primary enamel knots, whereas expression could not be detected in MS or R2 (Fig. 1P,T; Fig. S1). Weak expression of *Fgf16* and *Fgf17* were observed in cervical loop epithelium and mesenchyme surrounding the cervical loop, whereas MS or R2 showed no expression of these molecules (Fig. 1Q,R). *Fgf18* expression was detected in buccal and lingual mesenchyme but was absent from mesenchyme underneath cap epithelium (Fig. 1S). *Fgf21*, *Fgf22* and *Fgf23* showed no expression in tooth germs at E14.5 (data not shown). Strong expression of both *Etv4* and *Etv5* was detected in mesenchyme and epithelium but was absent from the primary enamel knots, whereas both have been shown to be weakly expressed in MS and R2 (Fig. 2G,H; Klein et al. 2006). *Fgf3* was expressed in mesenchyme and enamel knots, whereas *Fgf4* and *Fgf9* showed restricted expression only in primary enamel knots at this stage (Kettunen & Thesleff, 1998; Kettunen et al. 2000). *Fgf10* expression was observed in tooth mesenchyme (Kettunen et al. 2000). *Fgfr1IIIb* (receptor for Fgf3, Fgf10) was expressed in inner enamel epithelium, whereas *Fgfr1IIIc* (receptor for Fgf4, Fgf15, Fgf17, Fgf20) expression was found in tooth mesenchyme and inner enamel epithelium. *Fgfr2IIIb* (receptor for Fgf3, Fgf10, Fgf15) was expressed in outer epithelium and the stellate reticulum, whereas weak expression of *Fgfr2IIIc* (receptor for Fgf4, Fgf9, Fgf15, Fgf16, Fgf17, Fgf18 and Fgf20) was observed in buccal tooth mesenchyme at this stage (Kettunen et al. 1998; Mason, 2007). Fgf signalling is thus activated in inner enamel epithelium by Fgf3, Fgf4, Fgf10, Fgf15, Fgf17 and Fgf20 through *Fgfr1IIIb* and *Fgfr1IIIc*, in outer enamel epithelium and the stellate reticulum by Fgf3, Fgf10 and Fgf15 through *Fgfr2IIIb*, and in mesenchyme by Fgf4, Fgf9, Fgf15, Fgf16, Fgf17, Fgf18 and Fgf20 through *Fgfr1IIIc* and *Fgfr2IIIc*.

Fgf signalling was activated in the cells surrounding the primary enamel knot but not in the primary enamel knot cells, although *Fgf3*, *Fgf4*, *Fgf9*, *Fgf15* and *Fgf20* were expressed in the primary enamel knot at E14.5.

#### Cytodifferentiation (E18.5)

The terminal differentiation of dentine-forming odontoblasts from dental papilla cells and the enamel-forming ameloblasts from the internal epithelium occurred between E18 to P0. Primary enamel knots were transient and had disappeared by the late cap stage (E15 in mice). In the molars, however, secondary enamel knots developed that are clearly visible from expression of *Fgf4* and *Slit1* at the bell stage at the tip of the forming cusps (E18; Jernval et al. 1998; Pispá et al. 1999; Loes et al. 2001).

Expression of *Fgf20* was found in secondary enamel knots, whereas *Fgf15* was expressed in mesenchyme directly underneath the secondary enamel knots (Fig. 1U,Y). *Fgf16* expression was observed in ameloblasts (Fig. 1V). *Fgf17*, *Fgf18*, *Fgf21*, *Fgf22* and *Fgf23* showed no expression in tooth germs at this stage (Fig. 1W,X; data not shown).

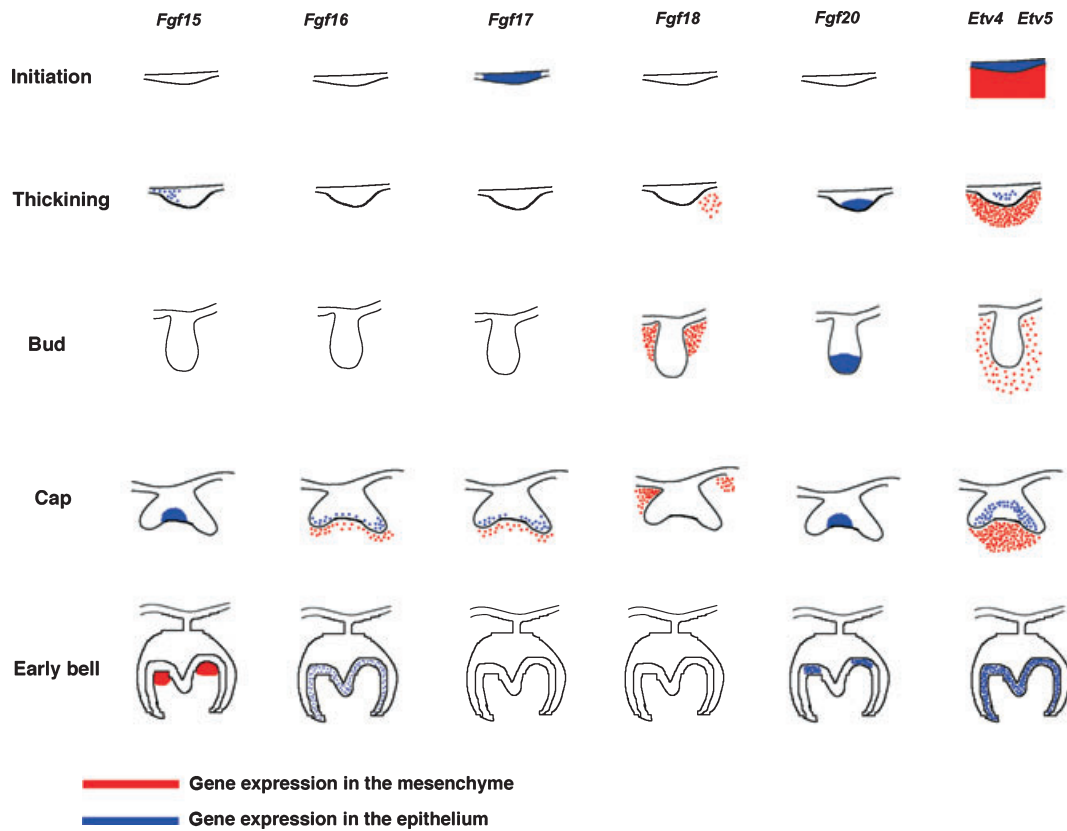
Strong expression of both *Etv4* and *Etv5* was observed in ameloblasts, whereas they were weakly expressed in odontoblasts (Fig. 2I,J). *Fgf3* was expressed in the dental papilla but was absent from terminally differentiated odontoblasts, whereas *Fgf10* was expressed in differentiating odontoblasts at this stage (Kettunen et al. 2000). *Fgf4* was also expressed in secondary enamel knots, whereas *Fgf9* was expressed in ameloblasts at E18.5 (Kettunen & Thesleff, 1998). *Fgfr1IIIb* (receptor for Fgf10) expression was observed in both odontoblasts and ameloblasts (Kettunen et al. 1998; Mason, 2007). *Fgfr1IIIc* (receptor for Fgf4, Fgf15 and Fgf20) was strongly expressed in odontoblasts but weakly expressed in ameloblasts (Kettunen et al. 1998; Mason, 2007). *Fgfr2IIIb* (receptor for Fgf10 and Fgf15) expression was detected in ameloblasts, and *Fgfr2IIIc* (receptor for Fgf4, Fgf9, Fgf15, Fgf16 and Fgf20) showed restricted expression in the dental follicle (Kettunen et al. 1998; Mason, 2007). In ameloblast development, Fgf signalling was activated by Fgf4, Fgf10, Fgf15 and Fgf20 through *Fgfr1IIIb*, *Fgfr1IIIc* and *Fgfr2IIIb*, and in odontoblasts by Fgf4, Fgf10, Fgf15 and Fgf20 through *Fgfr1IIIb* and *Fgfr1IIIc*. Mice with epithelial conditional mutation of *Fgfr1* show abnormal enamel formation due to abnormal enamel-secreting ameloblasts, suggesting that Fgf4, Fgf10, Fgf15 and Fgf20 play critical roles in ameloblast function (Takamori et al. 2008).

The expression of Fgf ligands, *Etv4* and *Etv5* during murine molar tooth development is summarised in Fig. 3. *Etv4* and *Etv5* were expressed in an almost identical manner in embryonic molar tooth development.

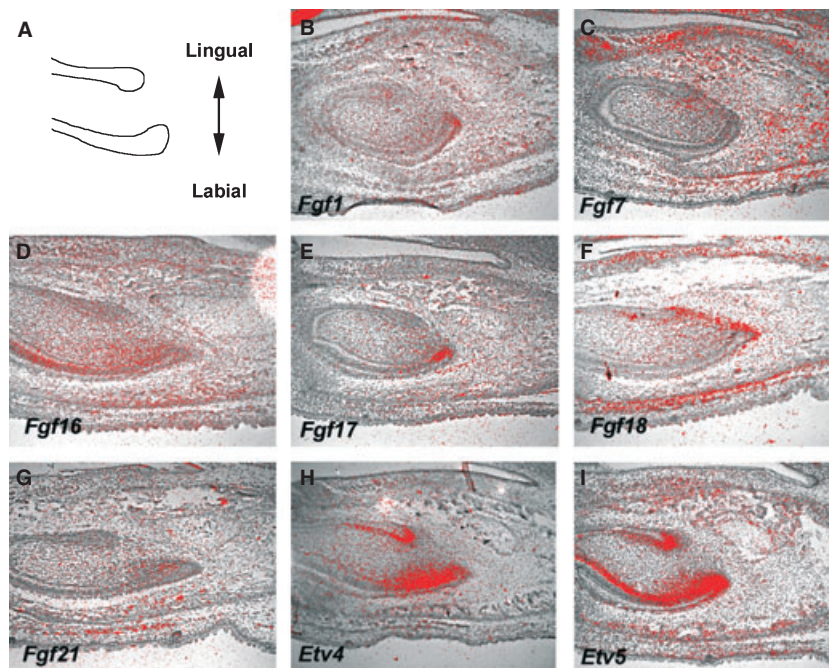
#### Cervical loops of incisors (E16.5)

Murine incisors grow continuously throughout life. The cervical loop region of mouse incisors is an epithelial stem cell niche that provides epithelial cell precursors for continuous growth. Labial and lingual cervical loops are different because rodent incisors have two distinct surfaces – a labial surface of enamel-covered dentine and a lingual surface of dentine only. *Fgf10* mutant mice have a compromised cervical loop formation (Harada et al. 2002). Mice with mutations in *Sprouty2/4* (antagonists of Fgf signalling) show bilateral enamel deposition due to disrupted inactivation of Fgf signalling in lingual cervical loops (Klein et al. 2008). Fgf signalling thus plays a critical role in the formation and function of cervical loops (Harada et al. 1999, 2002; Wang et al. 2007; Klein et al. 2008). Expression of only three Fgf ligands (*Fgf3*, *Fgf9* and *Fgf10*) has been described in cervical loops (Harada et al. 2002; Wang et al. 2007; Klein et al. 2008). We therefore examined the expression of other Fgf ligands in incisor cervical loop regions at E16.5 when the cervical loops can be observed as distinct structures.

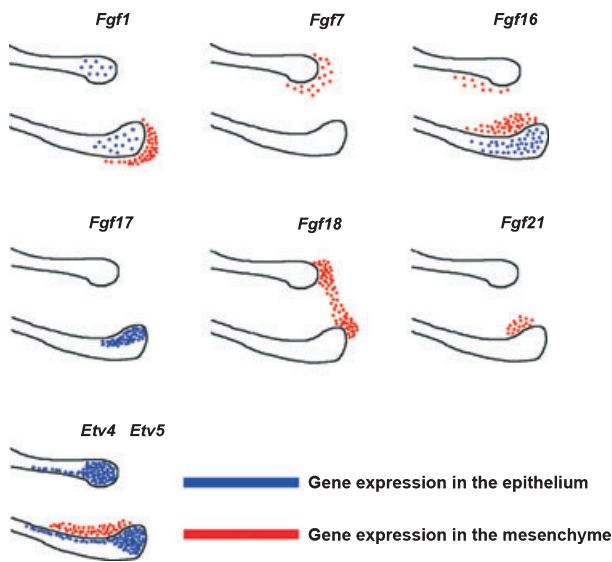
*Fgf1* was expressed in all cervical loop epithelia (Fig. 4A). Weak expression of *Fgf7* was found in mesenchyme close to lingual cervical loops (Fig. 4C). Strong *Fgf16* expression was



**Fig. 3** Summary of *Fgfs*, *Etv4* and *Etv5* in molar tooth development. Schematic representation of a combination of expression of *Fgf* ligands, *Etv4* and *Etv5*, in molar tooth development at E10.5, E12.5, E13.5, E14.5 and E18.5. *Etv4* and *Etv5* were expressed in an identical manner in embryonic tooth development. Expression in epithelium shown in blue and in mesenchyme in red.



**Fig. 4** Expression of *Fgf* ligands *Etv4* and *Etv5* in cervical loop region at E16.5. (a) Schematic representation of mouse incisor cervical loop at E16.5 and axial definition. (B–I) *In situ* hybridisation of *Fgf* ligands *Etv4* and *Etv5* on sagittal head sections at E16.5.



**Fig. 5** Summary of *Fgf* ligands *Etv4* and *Etv5* in embryonic cervical loop region. Schematic drawing of a combination of expression of *Fgf* ligands *Etv4* and *Etv5* in cervical loop region at E16.5. *Etv4* and *Etv5* were expressed in an identical manner in embryonic cervical loops. Expression in epithelium shown in blue and in mesenchyme in red.

observed in lingual mesenchyme close to labial cervical loops, whereas *Fgf16* was weakly expressed in labial cervical loop epithelium and labial mesenchyme close to lingual cervical loops (Fig. 4D). *Fgf17* expression was localised in labial cervical loop epithelium (Fig. 4D). *Fgf18* showed localised expression in mesenchyme between labial and lingual cervical loops (Fig. 4F). *Fgf21* was weakly expressed in lingual mesenchyme close to labial cervical loops (Fig. 4G). *Fgf2*, *Fgf4–Fgf6*, *Fgf8*, *Fgf15*, *Fgf19*, *Fgf20*, *Fgf22* and *Fgf23* expression could not be detected in cervical loop epithelium or incisor mesenchyme (data not shown). *Etv4* and *Etv5* showed strong expression in both labial and lingual cervical loop epithelia and lingual mesenchyme close to labial cervical loops (Fig. 4H,I; Klein et al. 2008). *Fgf3* expression was observed in labial mesenchyme close to cervical loops, whereas *Fgf9* expression was localised in a small lingual epithelial domain just anterior to the labial cervical loops (Harada et al. 1999; Wang et al. 2007; Klein et al. 2008). *Fgf10* has been shown to be expressed in mesenchyme close to both lingual and labial cervical loops (Harada et al. 1999; Klein et al. 2008). *Fgfr1b* and *Fgfr2b* were both expressed in cervical loop epithelium (Harada et al. 1999). It is likely that Fgf signalling is activated in cervical loop epithelium by *Fgf1*, *Fgf3*, *Fgf7*, *Fgf10* and *Fgf21* through *Fgfr1b* and *Fgfr2b*.

The expression of *Fgf* ligands *Etv4* and *Etv5* in murine embryonic incisor cervical loop regions is summarised in Fig. 5. *Etv4* and *Etv5* were expressed in an almost identical manner in embryonic cervical loop development.



**Fig. 6** *Fgf15* expression in development of craniofacial organs. *In situ* hybridisation of *Fgf15* on frontal head sections (A,B,D,E) and sagittal sections (C) at E13.5 (B,E) and E14.5 (A,D). Upper incisor (A), lower incisor (B,C), whisker hair follicles (D) and tongue (E).

### Tooth development in *Fgf15*<sup>-/-</sup> and *Fgf15*<sup>-/-</sup>;*Fgf8*<sup>+/-</sup> mice

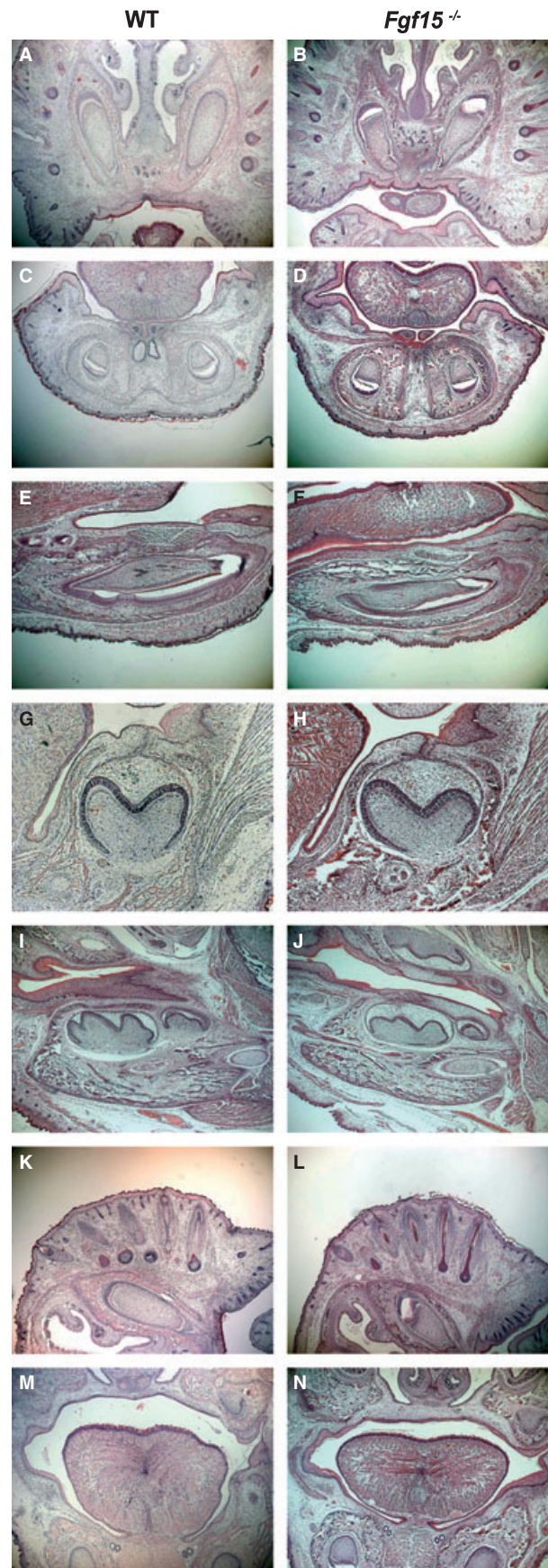
Fgf ligands show dynamic temporo-spatial expression in embryonic tooth development. Tooth abnormalities are found in mice with mutation in several Fgf signalling-related molecules (De Moerloose et al. 2000; Harada et al. 2002; Klein et al. 2006, 2008; Hosokawa et al. 2009).

*Fgf15* shows restricted expression from the early stages in molar tooth development. Restricted expression pattern of *Fgf15* is also observed in incisor tooth development before cervical loop formation (Fig. 6A–C). *Fgf15* was co-expressed with *Shh* in the brain and in primary enamel knots (Dassule et al. 2000; Gritli-Linde et al. 2002; Saitu et al. 2005; Gimeno & Martinez, 2007; Komada et al. 2008). To investigate the role of *Fgf15* in tooth development, we examined teeth from *Fgf15* mutant mice (*Fgf15*<sup>-/-</sup>). Most *Fgf15*<sup>-/-</sup> mice die the first week after birth (Vincentz et al. 2005). No significant abnormalities, however, could be detected in either incisors or molars in the mutant mice at birth (Fig. 7B,D,F,H,J). *Fgf15* expression is also observed in the development of other craniofacial organs such as the tongue and whisker follicles (Fig. 6D,E). However, no significant abnormalities were detected in these organs (Fig. 7L,N).

There is an overlap between *Fgf8* and *Fgf15* expression in tooth development at E12 (Fig. 1; Kettunen et al. 2000) and it is conceivable that *Fgf8* compensates for loss of *Fgf15* in mutant tooth development. To reveal any genetic interaction between *Fgf15* and *Fgf18*, we analysed teeth in *Fgf15*<sup>-/-</sup>;*Fgf8*<sup>+/-</sup> mice, but no obvious abnormalities could be detected in either incisors or molars in these mutant mice at birth (Fig. 8).

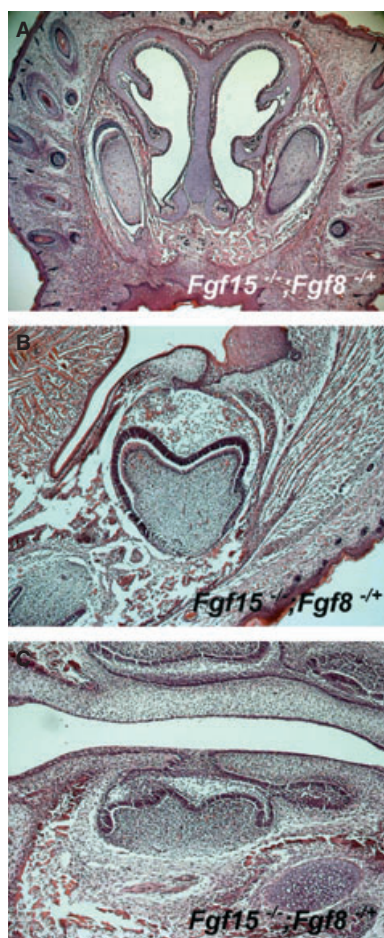
*Fgf9* and *Fgf20* are expressed in epithelium and *Fgf10* and *Fgf18* in mesenchyme when *Fgf15* expression is found in epithelium at E12.5 (Kettunen & Thesleff, 1998; Kettunen et al. 2000). In common with *Fgf15*, *Fgf3*, -4, -9 and -20 are expressed in the primary enamel knots at E14.5 (Kettunen & Thesleff, 1998; Kettunen et al. 2000). It is conceivable that there is multiple functional redundancy between these ligands in tooth development. *Fgf8*, *Fgf10* and *Fgf20* can share receptors with *Fgf15* at E12.5, whereas *Fgf3*, *Fgf4*, *Fgf9* and *Fgf20* expressed in enamel knots also share receptors with *Fgf15* at E14.5.

Expression of all Fgf ligands has been examined at embryonic stages of tooth development, although *Fgf5*, *Fgf6* and *Fgf23* could not be detected in tooth development during these embryonic periods (Kettunen & Thesleff, 1998;



**Fig. 7** Teeth, whisker hair follicles and tongue in *Fgf15*<sup>-/-</sup> mice. No obvious abnormalities are found in upper (B) or lower (D,E) incisors, and molars (H,J) in *Fgf15*<sup>-/-</sup> mice. No significant morphological changes are observed in whisker hair follicles (L) and tongue (N) in *Fgf15*<sup>-/-</sup> mice. Frontal (A–D,G,H,K–N) and sagittal (E,F,I,J) head sections at birth.





**Fig. 8** Teeth in *Fgf15*<sup>-/-</sup>;*Fgf8*<sup>+/-</sup> mice. No obvious abnormalities are found in upper (A) and molars (B,C) in *Fgf15*<sup>-/-</sup>;*Fgf8*<sup>+/-</sup> mice. Frontal (A,B) and sagittal (C) head sections at birth.

Kettunen et al. 2000). However, *Fgf23* expression was observed at postnatal stages of tooth development (Onishi et al. 2008). Moreover, overexpression of *Fgf23* results in the hypo-mineralisation of teeth (Onishi et al. 2008).

The dynamic temporo-spatial expression of Fgf ligands, their receptors and Fgf signalling target molecules shows that Fgf activity regulates multiple aspects of tooth development. There may be multiple functional redundancy between these ligands in tooth development.

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## Supporting Information

Additional Supporting Information may be found in the online version of this article:

**Fig. S1** *Fgf15* and *Fgf20* expression in MS or RS at E13.5 or E14.5. Frontal head sections showing no obvious expression of *Fgf20* in MS of wild-type at E13.5 (A–C). Frontal head sections showing no obvious expression of *Fgf15* (D–F) or *Fgf20* (G) in MS or RS of wild-type at E14.5 (D–G).

**Table S1** Tooth phenotypes in mice with mutation of Fgf signaling-related molecules.

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